Mol Gen Genet (1983) 189:370–374

MGG

Inheritance of the rDNA Spacer in *D. melanogaster*

E. Boncinelli,[1] A. Borghese,[1] F. Graziani,[1] G. La Mantia,[2] A. Manzi,[1] C. Mariani,[1] and A. Simeone[1]

[1] International Institute of Genetics and Biophysics, C.N.R., via Marconi, 10, Naples, Italy

[2] Istituto di Biologia Generale e Genetica, via Mezzocannone, 8, Naples, Italy

Summary. We studied the organization of rDNA spacer sequences within several rDNA loci in *D. melanogaster*. Every locus showed many discrete length classes of rDNA spacer, ranging from 2.4 kb to about 20 kb. Different loci show characteristic distributions of spacers within the various length classes. Using this molecular characteristic as a genetic marker, segregation, recombination and occurrence of unequal crossing-over were studied. The rDNA loci segregated with a Mendelian pattern; interhomologous recombination events occurred at the expected rate; conversely unequal crossing-over within the rDNA locus appears not to be as frequent as expected.

Introduction

In *Drosophila melanogaster* the genes coding for 28 S and 18 S ribosomal RNA (rDNA) are located in the heterochromatic region of the X chromosome and on the short arm of the Y chromosome (Ritossa et al. 1966; Cooper 1959). Each gene has a transcribed region and a nontranscribed one called rDNA spacer. Nontranscribed spacers (rDNA NTS) have been reported to be heterogeneous in length within different loci (Dawid et al. 1978; Wellauer et al. 1978; Indik and Tartof 1980). Restriction analysis of the spacer structure of cloned rDNA gene units derived from wild-type flies (Oregon R) indicates that spacers are internally repetitious, and that the observed length heterogeneity derives, at least in part, from a number of internal repeated sequences (Long and Dawid 1979).

The study of the organization of the spacers in general (Fedoroff 1979) and rDNA spacers in particular (Wellauer et al. 1976) has proved to be extremely interesting from several points of view.

One of these is the so-called horizontal (or concerted) evolution of repeated gene familes (Smith 1973; Tartof 1974; Brown and Sugimoto 1973). Individual members of the rDNA multigene family within a single species are far more similar to each other than would be expected if each gene had evolved independently of other family members. This fact is especially surprising for gene portions that have no known function, e.g. spacers. Such regions would be expected to accumulate mutational differences and exhibit variation within a single species. In fact different species show a very low level of sequence homology in the rDNA spacers (for a recent review see Dover 1982).

One model that can account for the concerted evolution of multigene familes involves unequal crossing-over among the tandemly arranged members of the gene family (Smith 1973), provided that the rate of crossover fixation will be high enough to counteract the frequence of genetic variation within the locus.

With this in mind we undertook an analysis of the organization and mode of inheritance of the rDNA spacer in wild-type and mutant loci of *D. melanogaster* using spacer length heterogeneity as a marker. In fact each locus shows a characteristic distribution of length classes of spacer that can be followed through the generations.

The segregation of the rDNA locus has been analysed by observing the distribution of spacers in the progeny of female flies, heterozygous for the X-linked rDNA loci. We found that the rDNA locus segregates as a Mendelian unit.

Moreover we have determined the rate of occurrence of unequal crossing-over within the rDNA locus. In order to do that a number of flies from appropriate crosses were analysed individually to test for interindividual variability that could be generated, inter alia, from sister strand unequal crossing-over of a both premeiotic and meiotic nature.

Materials and Methods

Drosophila Stocks. O−R is wild-type Oregon-R from the University of Naples. C−S is wild-type Canton-S. B4 is *v f su(f)* from Oak Ridge. ♀♀B6 are C(1)DX, *y f* and ♂♂B6 are g^2 *ty bb*, both originally from Pasadena. ♀♀B30 are C(1)DX, *y f* and ♂♂B30 are R(1)2, *y B*, both from Oak Ridge; ♀♀E2 hom. are *w sn*. ♀♀F34 are C(1)RM, *y w* from the Chicago collection. ♀♀F27 is XY, y^2 *su*(w^a) w^a. Most of these stocks can be considered isolates, because they have been established for 60 years or more. Furthermore, the composition of several stocks is such that males and females exchange only their Y chromosome, whereas the X chromosome of males and the attached X's of the females remain physically separated.

DNA Extraction. Cloned rDNA fragments cDmra56, cDmrY22, cDmre52, were obtained from P.K. Wellauer. cDm4G3 was obtained from W. Gehring. pDmHAII was

* *Present Address:* EMBL, Postfach 102240, D-69 Heidelberg 1

Offprint requests to: E. Boncinelli

obtained by subcloning a *Hin*dIII – *Hae*III spacer fragment of cDmra56 in a pBR322 derivative. All cloned rDNA fragments were prepared as described in Dawid et al. (1978). rDNA fragments were nick-translated according to Rigby et al. (1977). DNA from about 5 g adult flies was prepared as previously described by Ritossa et al. (1971). Its molecular weight was about 60 kilobases (kb). DNA from a single adult fly was prepared by pronase-EDTA-SDS lysis at 65° C (Kavenoff and Zimm 1973) followed by one phenol extraction. After an exhaustive dialysis against Tris-HCl 10 mM pH 8.0, EDTA 1 mM (TE buffer), the solution was brought to 0.3 M Na-Acetate and the DNA precipitated with 2 volumes of cold ethanol with 0.5 µg λDNA as a carrier.

Restriction and Hybridization. Restriction endonuclease digests of total DNA were performed according to the manufacturer's instructions (BRL, New England Biolabs, Boston, USA and Miles Slough, England). DNA fragments were separated by electrophoresis on agarose gel in Tris-Borate-EDTA buffer. Blotting was according to Southern on S & S nitrocellulose filters (Southern 1975). Filters were preincubated in 3 × SSC, 0.1% SDS, 5 × Denhart solution (1 × is 0.02% Ficoll, 0.02% Polyvinyl Pyrrolidone, 0.02% Bovine Serum Albumin) (Denhart 1966) for a least 6 h 65° C and hybridized for 24 h in 1 × Denhart solution, 3 × SSC, 0.1% SDS, plus 10 ng heat-denatured P^{32}-labeled probe. After repeated washing in 3 × SSC, 0.1% SDS, at 65° C, filters were rinsed with 0.2 × SSC, dried under vacuum and exposed to flash activated autoradiographic film (Kodak 5R) using an intensifying screen (Laskey and Mills 1979). To quantitate our data, before digestion we added to each DNA sample, increasing quantities of purified DNA from a cloned repeat containing an NTS of a length comparable to that of the rDNA bands under scrutiny. The autoradiographic intensities of the bands were compared to the standard; from the quantity of plasmid DNA added, the number of gene copies per genome can be computed and, from this, the relative multiplicity of the various spacer classes in each stock. The sensitivity of the technique enables measurement of well below one spacer unit per genome.

Results

Comparision of rDNA Spacer Pattern of Different Stocks

A physical map of *D. melanogaster* ribosomal genetic units is shown in Fig. 1 (Wellauer and Dawid 1978; Glover and Hogness 1977). As indicated, endonuclease *Hin*dIII cleaves all clones at the 5′-end of the nontranscribed spacer, whereas the *Hae*III cut is at two-thirds of the so-called external transcribed spacer (ETS), 250 bp upstream of the 5′-end of the 18 S transcribing region. The total DNA extracted from adult flies of several stocks was digested with these two enzymes; after the separation of various fragments on agarose gel and transfer onto nitrocellulose filter this was hybridized with a radioactive probe of the cloned rDNA spacer, pDmHAII. Figure 2 shows the resulting autoradiographic pattern. Spacer bands are observed from 2.4 kb to about 20 kb. Many bands can be seen in each stock analysed and the size of some common bands corresponds to reported *Hin*dIII – *Hae*III rDNA spacer fragments of

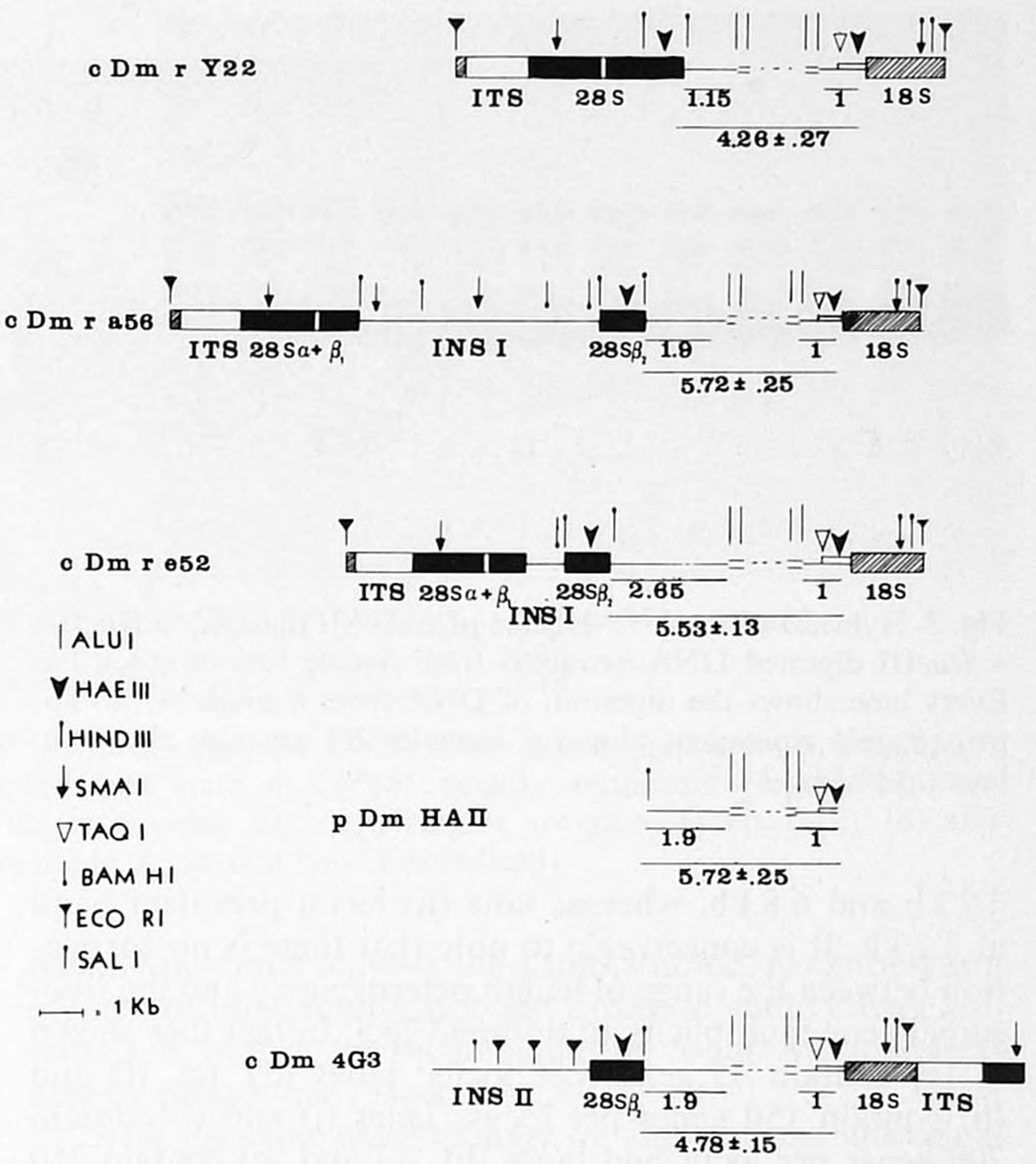

Fig. 1. Schematic representation of the rDNA units inserted in the plasmids used as radioactive probes. Full boxes represent the 28S genes; etched boxes represent 18S genes and open boxes the internal transcribed spacers (ITS). INS I means type I rDNA insertion, INS II means type II rDNA insertion. The narrower open boxes upstream of 18S genes represent external transcribed spacer (ETS). The spacer length – from *Hin*dIII site to *Hae*III site – is given ± standard error in kilobases (kb). The internal, repeated region of spacers is not to scale. cDm 4G3 contains only part of a unit. pDm HAII was subcloned from cDmr a 56; it is shown as an example of the four subcloned spacers

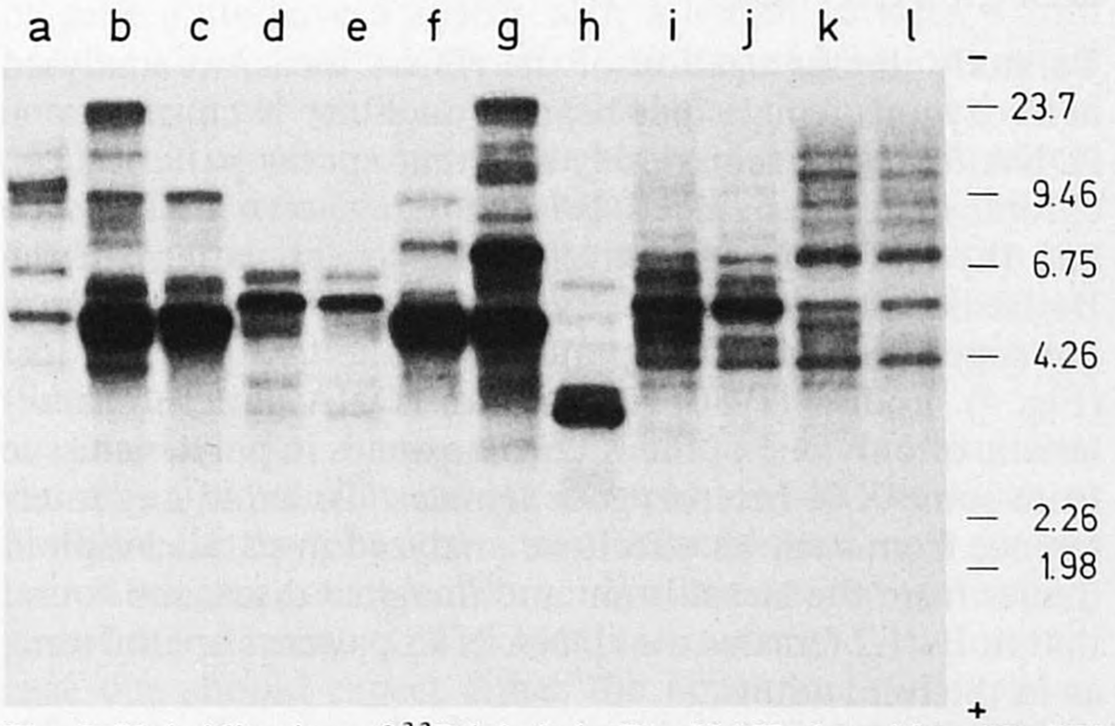

Fig. 2. Hybridization of ^{32}P-labeled pDm HAII plasmid to *Hin*dIII – *Hae*III digested total DNA (0.5 µg) extracted from the following types of flies: (a) ♀♀ F27; (b) ♀♀ O–R; (c) ♂♂ O–R; (d) ♀♀ C–S; (e) ♂♂ C–S; (f) ♀♀ B4; (g) ♀♀ F34; (h) ♀♀ E2; (i) ♀♀ B6; (j) ♂♂B6; (k) ♀♀ B30; (l) ♂♂B30. Standard lengths are given in kb

cloned rDNA units (Long and Dawid 1979). However a striking difference can be observed regarding the ralative intensity of the rDNA spacer classes within every lane. As an example, lane (g), of Fig. 2 shows prevalent bands at 4.4 kb and 6.9 kb; lane (l) shows strong bands at 3.9 kb,

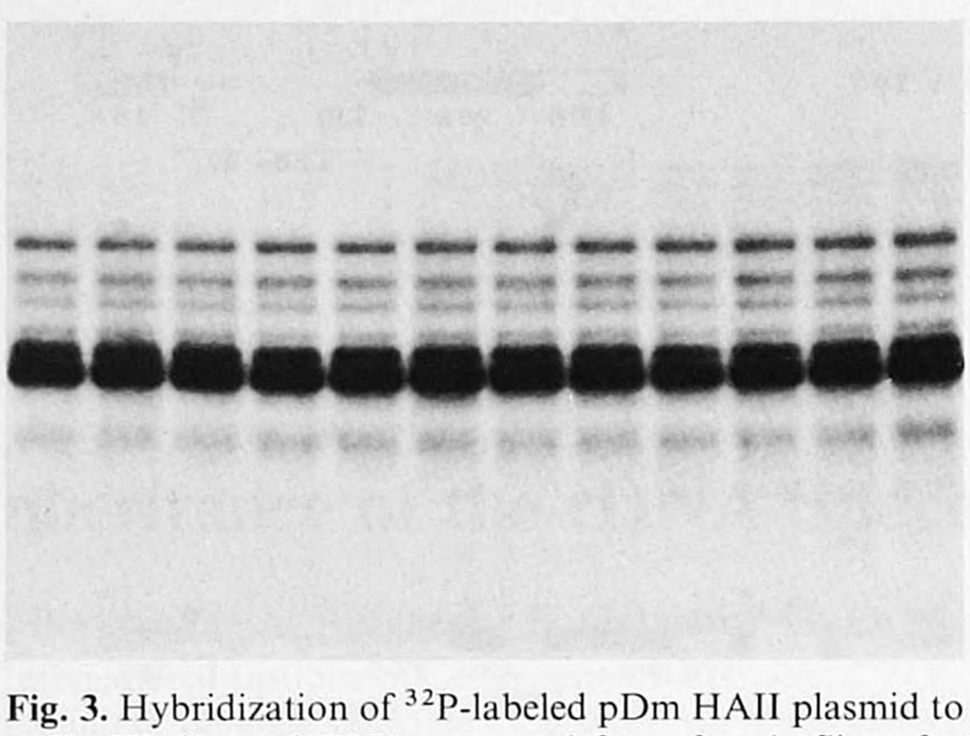

Fig. 3. Hybridization of ^{32}P-labeled pDm HAII plasmid to *Hin*dIII – *Hae*III digested DNA extracted from female flies of stock E2. Every lane shows the digestion of DNA from a single fly, in approximately equivalent aliquots. Females E2 are also shown in lane (h) of Fig. 2

5.2 kb and 6.8 kb, whereas lane (h) has a prevalent band at 3.2 kb. It is conceivable to note that there is no correlation between the range of length heterogeneity and the ribosomal gene multiplicity of different loci. In fact flies shown in (a) contain 45 genes per locus; lanes (d), (e), (f) and (h) contain 150 genes per locus; lanes (j) and (l) contain 200 genes per locus and lanes (b), (c) and (g) contain 250 genes per locus (Ritossa 1976 and unpublished results).

The observed rDNA spacer patterns are actual individual patterns, and do not arise as a population average. In fact we repeated the same experiment using DNA from single flies, and we obtained the same patterns; both qualitatively and quantitatively. As an example see in Fig. 3 patterns of single homozygous female flies from the stock shown in lane (h) of Fig. 2.

Inheritance of rDNA Spacer Patterns in Single Fly Crosses

To study the segregation of the rDNA locus, we analysed heterozygous female flies bearing on either X chromosome rDNA loci with remarkably different spacer patterns, i.e., chromosomes designated B4 and E2, shown in lanes (f) and (h) of Fig. 2, respectively. The spacer pattern of the B4/E2 heterozygote is exactly what could be expected from the superposition of the patterns for the two parental loci (Fig. 4). Endow (1980) reported an rDNA pattern characteristic of only one of the X chromosomes in polytene tissue from some X/X heterozygous females. To avoid any interference from such an effect, we analyzed in parallel diploid tissues from the larval brain and imaginal discs, and found that in B4/E2 females the rDNA NTS patterns are the same as in the total adult.

One heterozygous female was then crossed with wild-type males to obtain male progeny hemizygous for the X chromosome derived from the mother. Ninety seven such males were mated singly to $\widehat{XX}/Ybb^-$ females to establish new stocks. X/Ybb^- males from each stock so obtained were analysed for their rDNA NTS pattern. This genetic composition was chosen because these males show only the pattern pertaining to the X-linked rDNA locus; the Ybb^- chromosome in fact does not contribute ribosomal genes. Males from 47 new lines showed an E2 parental pattern, males from 49 new lines showed a B4 parental pattern; males from one established line showed a recombinant pattern (Fig. 4). The pattern of these new stocks proved to be very stable through the following generations. The band pattern of the recombinant male indicates that the recombinant locus might have arisen in one of two ways. The crossing-over might have occurred near one end of both loci, maintaining practically all the E2 units and losing the B4 units. Alternatively, assuming that in both parental loci, NTS classes of similar length are clustered and not randomly intermingled, the exchange might have occurred in a more median region of the locus. Unequal crossing can, in this case, be excluded, both on the basis of the pattern features and because the rDNA redundancy of the loci remained unchanged: the saturation level of rDNA (% of total DNA) is 0.155 ± 0.010, 0.160 ± 0.009 and 0.160 ± 0.008, respectively for E2 parental, B4 parental and recombinant X chromosome.

Intrastock Variability of rDNA Spacer Patterns

The rate of unequal meiotic and premeiotic sister chromatid exchange can be evaluated by analysing individual spacer distributions of single flies carrying the same X-linked locus. We studied in four different genetic crosses the rDNA locus of the *w sn* X chromosome whose spacer length pattern is shown in lane (h) of Fig. 2. Originally this chromosome was present in males of stock A: $\widehat{XX}/Ybb^-$ and X, *w sn*/Ybb^-. Here the only possible exchange within the rDNA locus is sister chromatid crossing-over in males. In fact the chromosome under scrutiny is only present in males where the homologue carries a deletion.

Six years (about 150 generations) before the present investigation a new stock was established from stock A: X, *w sn*/X, NO^- and X, *w sn*/Ybb^- (stock B). In this cross the X-linked locus has a deleted homologue both in females and males.

In stock C ($\widehat{XX}/Ybb^+$ and X, *w sn*/Ybb^+) the rDNA locus could in principle recombine with the wild-type homologue in males, but in *D. melanogaster* the recombination in males is extremely low.

Finally in stock D (X, *w sn*/X, *w sn* and X, *w sn*/Ybb^+) the locus can recombine without constraints in female homozygotes.

We individually analyzed 600 random males from 40 generations of stock A; 400 males from 20 generations of stock B; and about 300 males from 20 generations of each of stocks C and D.

No variant was observed, which puts an upper limit of 10^{-5} per cell generation on the frequency of unequal crossing-over within the rDNA locus of this chromosome.

Discussion

We studied the arrangement of the rDNA spacer units in and within different stocks of *D. melanogaster*. We digested total DNA of adults of several genetic compositions with endonucleases *Hin*dIII and *Hae*III simultaneously, transferring it to filters after separation on agarose gel and hybridizing it with a radioactive probe containing the rDNA spacer. Several discrete bands can be observed in every stock as a consequence of an extended length heterogeneity of spacers within each locus. Different length classes are unevenly represented within a locus. The multiplicity of various classes can range from 1–150, as can be deduced from

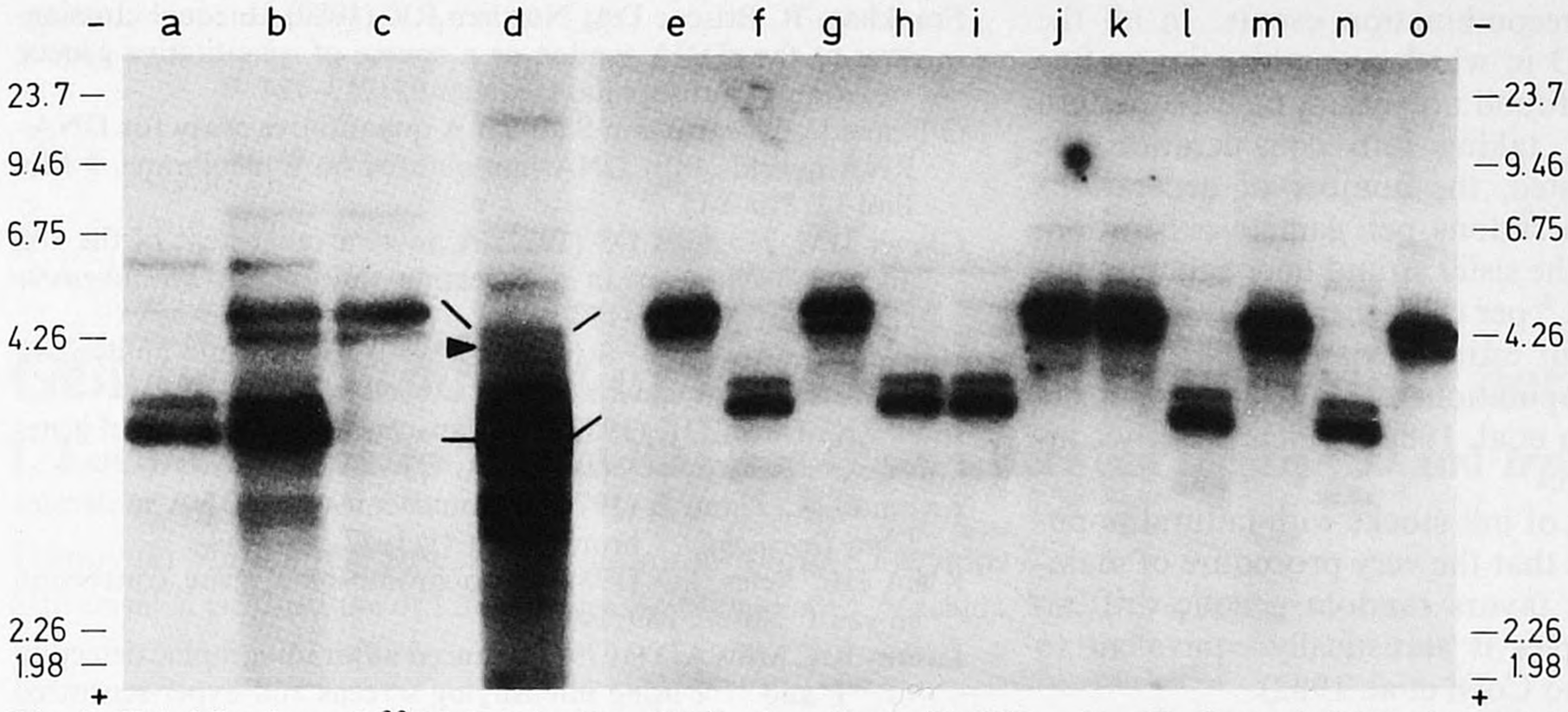

Fig. 4. Hybridization of ^{32}P-labeled pDm HAII plasmid to *Hin*dIII – *Hae*III digested DNA from: (a) an E2 parental female; (b) an E2/B4 heterozygous female; (c) a B4 parental female; (d) a recombinant male of X/Y*bb*$^-$ genetic composition segregated from the E2/B4 heterozygous female (e–o) nonrecombinant males from the same cross. Standard lengths are given in kb. (a–c), (d) and (e–o) are from different gels. (d) is overexposed to show the triplet containing the variant band (arrowhead)

the relative intensity of hybridization bands in the same lane. The band pattern and the relative intensity relationship remains unaltered if hybridization is to rDNA spacer sequences subcloned from different rDNA units shown in Fig. 1 (data not shown). The homology in sequence turns out to be very high among different spacer repeats and various loci. The distribution of rDNA spacers in length classes is, in contrast, unique for each locus and can be considered a characteristic molecular marker.

For every genetic combination we investigated, single individuals were also analyzed (see Fig. 3). In each case the individual pattern coincided with that obtained from a pool of flies of the same stock, thus the patterns shown in Fig. 2 are individual patterns, and not population averaged artifacts.

The spacer classes longer than ten kilobases require special mention.

They are absent in wild-type Canton-S and in most of the stocks examined, although they have been observed in stock F34 and wild-type Oregon-R flies. Similar observations using Oregon-R flies have been reported by Indik and Tartof (1980) who speculate on the possible role of long spacers in the magnification process (Ritossa 1976).

Nevertheless, many loci lacking very long spacer classes are capable of undergoing a magnification process.

Using the characteristic rDNA spacer patterns as markers, we investigated segregation and recombination of the two rDNA loci carried by two X chromosomes. The chosen loci were easily distinguished by means of their NTS patterns even though they carry a similar number of rDNA units. The heterozygous female exhibits a pattern containing bands of both parental chromosomes with the expected relative intensities (Fig. 4). A 1:1 segregation ratio of parental loci was observed, with one recombinant among 97 single chromosomes examined.

Our experiment was not designed to give a reliable estimate of recombination frequencies within the rDNA locus.

However, the finding is consistent with the reported recombination frequency in the locus (0.4%–0.6% in an inverted X, *sc*^{8} chromosome (Schalet 1969) and purported to be lower in a noninverted X chromosome (Schalet 1969; Hilliker et al. 1980). The earlier evaluation was based on phenotypic traits only: as the authors noted, recombination events not implying phenotypic variation could escape observation; in fact, the recombinant we found was undetectable by phenotypic inspection.

With the same methodology we analysed the individual rDNA spacer variation in appropriately designed stocks, in order to measure the rate of unequal crossing-over in rDNA loci. Every locus contains repeated gene units and every gene unit contains repeated sequences within the nontranscribed spacer. Accordingly the unequal crossing-over can occur owing to an imperfect pairing either of the gene units or of the subrepeats within spacers. In the latter case one should expect the appearance of a new spacer with a length varying from the original. The locus studied comprises only 11 length classes of comparatively short spacers. Furthermore their distribution is so asymmetrical that 75% of gene units have a spacer with a length between 3.2 kb and 3.3 kb. As the sensitivity of our technique is one single spacer unit per genome, we calculated that at least 95% of unequal crossing-over events between repeated sequences within the spacers would be detectable. It has to be noted, however, that this type of unequal crossing-over does not homogenize the locus and has not been considered as part of the model proposed to account for concerted evolution (Smith 1973).

On the other hand, the second type of crossing-over causes an expansion (and a contraction) of the locus as a whole. If the physical site of recombination is in two paired spacers one can observe, in addition, the same changes of the previous type of unequal crossing-over. In every case one should expect either the appearance (disappearance) of single length classes or an increase (decrease) in multiplicity of the existing length classes. To evaluate the exact probability of observing a crossover the power of resolution of the technique (which is easy to measure) must be known and the organization of the spacers in the locus, i.e., whether or not spacers of similar length are clustered, and that is not known. In general we can add that the probability of a given crossing-over taking place unnoticed is inversely proportional to its role in maintaining the gene family homogeneous.

The genetic combinations we chose for our analysis

allow different types of recombination events. In all the stocks – including stock D in which recombination is free from any constraint – we found no variant of NTS pattern distribution. Our results, taking into consideration the number of single flies tested, the number of generations and the value of cell generations per gamete generation, impose an upper limit on the sister strand unequal crossing-over of approximately 10^{-5} per cell generation.

One recent independent estimate of unequal crossing-over rate in *Drosophila* populations is 3×10^{-4} per gamete per generation (Frankham et al. 1980), which is fairly consistent with our evaluation.

As for the comparison of our stocks with natural populations, it should be noted that the very procedure of maintaining laboratory stocks favors random genetic drift so that a collection of samples is statistically equivalent to a large population (see also Coen et al. 1982).

Our results contrast to some extent with those obtained for yeast (Petes 1980; Szostak and Wu 1980). In that case, unequal sister chromatid exchange frequency has been estimed at 10^{-2} per generation during mitotic growth and even higher during meiosis. However, in yeast there is no length heterogeneity within the rDNA locus and yeast may have one or more mechanisms, in addition to those present in *Drosophila*, to constrain the internal variability of rDNA gene clusters; indeed recently intrachromosomal gene conversion in repeated genes of yeast has been observed (Klein and Petes 1981).

The newly established stocks from individual segregants of E2/B4 female heterozygotes turned out to be stable through generations and not internally polymorphic, although this point has not been so extensively verified as in the above-mentioned stocks. The stability of single stocks in contrast to the diversity between stocks, poses the problem of the origin of this diversity. Possibly new patterns may arise by discontinuous processes, one of which is certainly interchromosomal recombination.

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Communicated by Ch. Auerbach

Received October 20, 1982